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(54) Title: A NOVEL CLONED GABA-RECEPTOR SUBUNIT cDNA SEQUENCE AND STABLY CO-TRANSFECTED CELL LINES

(57) Abstract

The present invention relates to the cloning of a novel cDNA sequence encoding the ε receptor subunit of the $GABA_A$ receptor; to stably co-transfected eukaryotic cell lines capable of expressing a $GABA_A$ receptor, which receptor comprises the novel ε receptor subunit; and to the use of such cell lines in screening for and designing medicaments which act upon the $GABA_A$ receptor.

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A NOVEL CLONED GABA-RECEPTOR SUBUNIT cDNA SEQUENCE AND STABLY CO-TRANSFECTED CELL LINES

This invention concerns the cloning of a novel cDNA sequence encoding a particular subunit of the human $GABA_A$ receptor. In addition, the invention relates to a stable cell line capable of expressing said cDNA and to the use of the cell line in a screening technique for the design and development of subtype-specific medicaments.

Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system. It mediates fast synaptic inhibition by opening the chloride channel intrinsic to the $GABA_A$ receptor. This receptor comprises a multimeric protein of molecular size 230-270 kDa with specific binding sites for a variety of drugs including benzodiazepines, barbiturates and β-carbolines, in addition to sites for the agonist ligand GABA (for reviews see MacDonald and Olsen, *Ann. Rev. Neurosci.*, 1994, 17, 569; and Whiting *et al*, *Int. Rev. Neurobiol.*, 1995, 38, 95).

Molecular biological studies demonstrate that the receptor is composed of several distinct types of subunit, which are divided into four classes (α, β, γ and δ) based on their sequence similarities. To date, in mammals, six types of α (Schofield *et al., Nature (London)*, 1987, 328, 221; Levitan *et al., Nature (London)*, 1988, 335, 76; Ymer *et al., EMBO J.*, 1989, 8, 1665; Pritchett & Seeberg, *J. Neurochem.*, 1990, 54, 802; Luddens *et al., Nature (London)*, 1990, 346, 648; and Khrestchatisky *et al., Neuron*, 1989, 3, 745), three types of β (Ymer *et al., EMBO J.*, 1989, 8, 1665), three types of γ (Ymer *et al., EMBO J.*, 1990, 9, 3261; Shivers *et al., Neuron*, 1989, 3, 327; and Knoflach *et al*, *FEBS Lett.*, 1991, 293, 191) and one δ subunit (Shivers *et al., Neuron*, 1989, 3, 327) have been identified.

The differential distribution of many of the subunits has been characterised by *in situ* hybridisation (Shivers *et al., Neuron*, 1989, 3, 327; Wisden *et al*, *J. Neurosci.*, 1992, 12, 1040; and Laurie *et al*, *J. Neurosci*,

1992, 12, 1063) and this has permitted it to be speculated which subunits, by their co-localisation, could theoretically exist in the same receptor complex.

Various combinations of subunits have been co-transfected into cells to identify synthetic combinations of subunits whose pharmacology parallels that of *bona fide* $GABA_A$ receptors *in vivo* (Pritchett *et al.*, *Science*, 1989, 245, 1389; Pritchett and Seeberg, *J. Neurochem.*, 1990, 54, 1802; Luddens *et al.*, *Nature (London)*, 1990, 346, 648; Hadingham *et al*, *Mol. Pharmacol.*, 1993, 43, 970; and Hadingham *et al.*, *Mol. Pharmacol.*, 1993, 44, 1211). This approach has revealed that, in addition to an α and β subunit, either γ_1 or γ_2 (Pritchett *et al.*, *Nature (London)*, 1989, 338, 582; Ymer *et al.*, *EMBO J.*, 1990, 9, 3261; and Wafford *et al.*, *Mol. Pharmacol.*, 1993, 44, 437) or γ_3 (Herb *et al.*, *Proc. Natl. Acad. Sci. USA*, 1992, 89, 1433; Knoflach *et al.*, *FEBS Lett.*, 1991, 293, 191; and Wilson-Shaw *et al.*, *FEBS Lett.*, 1991, 284, 211) is also generally required to confer benzodiazepine sensitivity, and that the benzodiazepine pharmacology of the expressed receptor is largely dependent on the identity of the α and γ subunits present. Receptors containing a δ subunit (i.e. αβδ) do not appear to bind benzodiazepines (Shivers *et al.*, *Neuron*, 1989, 3, 327; and Quirk *et al.*, *J. Biol. Chem.*, 1994, 269, 16020). Combinations of subunits have been identified which exhibit the pharmacological profile of a BZ_1 type receptor ($\alpha_1\beta_1\gamma_2$) and a BZ_2 type receptor ($\alpha_2\beta_1\gamma_2$ or $\alpha_3\beta_1\gamma_2$, Pritchett *et al.*, *Nature (London)*, 1989, 338, 582), as well as $GABA_A$ receptors with a novel pharmacology, $\alpha_5\beta_2\gamma_2$ (Pritchett and Seeberg, *J. Neurochem.*, 1990, 54, 1802), $\alpha_4\beta_2\gamma_2$ (Wisden *et al*, *FEBS Lett.*, 1991, 289, 227) and $\alpha_6\beta_2\gamma_2$ (Luddens *et al.*, *Nature (London)*, 1990, 346, 648). The pharmacology of these expressed receptors appears similar to that of those identified in brain tissue by radioligand binding, and biochemical expperiments have begun to determine the subunit composition of native GABA receptors (McKernan & Whiting, *Tr. Neurosci.*, 1996, 19, 139). The exact structure

of receptors *in vivo* has yet to be definitively elucidated.

International Patent Specification No. WO 96/06862, published 7th March 1996 describes a human $GABA_A$ receptor epsilon subunit and nucleic acids encoding such polypeptides. The subunit of this publication has since been renamed as the pi (π) subunit (see Hedblom & Kirkness, *J. Biol. Chem.*, 1997, 272(24), 15346) and is unrelated to the epsilon subunit of the present invention.

The present invention relates to a new class of GABA receptor subunit, which we initially referred to as the kappa subunit (κ subunit) but will now hereinafter refer to as the epsilon subunit (ε subunit). Any inadvertant reference to kappa in this document will be understood to be synonymous with a reference to epsilon.

The nucleotide sequence for the epsilon subunit, together with its deduced amino acid sequence corresponding thereto, is depicted in Figure 1 of the accompanying drawings.

The present invention accordingly provides, in a first aspect, a DNA molecule encoding the epsilon subunit of the human GABA receptor comprising all or a portion of the sequence depicted in Figure 1, or a modified human sequence.

The term "modified human sequence" as used herein referes to a variant of the DNA sequence depicted in Figure 1. Such variants may be naturally occuring allelic variants or non-naturally occuring or "engineered" variants. Allelic variation is well known in the art in which the nucleotide sequence may have a substitution, deletion or addition of one or more nucleotides without substantial alteration of the function of the encoded polypeptide. Particularly preferred allelic variants arise from nucleotide substitution based on the degeneracy of the genetic code.

The sequencing of the novel cDNA molecules in accordance with the invention can conveniently be carried out by the standard procedure described in accompanying Example 1; or may be accomplished by alternative molecular cloning techniques which are well known in the art,

such as those described by Maniatis *et al.* in *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Press, New York, 2nd edition, 1989.

In a further aspect, the present invention also relates to polynucleotides (for example, cDNA, genomic DNA or synthetic DNA) which hybridize under stringent conditions to the DNA molecule depicted in Figure 1. As used herein, the term "stringent conditions" will be understood to require at least 95% and preferably at least 97% identity between the hybridized sequences. Polynucleotides which hybridize under stringent conditions to the DNA molecule depicted in Figure 1 preferably encode polypeptides which exhibit substantially the same biological activity or function as the polypeptide depicted in Figure 1.

The present invention further relates to a GABA epsilon subunit polypeptide which has the deduced amino acid sequence of Figure 1, as well as fragments, analogs and derivatives thereof.

The terms "fragment", "derivative" and "analog" when referring to the polypeptide of Figure 1, means a polypeptide which retains essentially the sme biological activity or function as the polypeptide depicted in Figure 1. Thus, an analog may be, for example, a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 may be one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residues may or may not be one encoded by the genetic code; or one in which one or more of the amino acid residues includes a substituent group; or one in which the mature polypeptide is fused with another compound,

such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol); or one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the technical capabilities of those skilled in the art.

The polypeptides and DNA molecules of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring DNA molecule or polypeptide present in a living animal is not isolated, but the same DNA molecule or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such DNA molecules could be part of a vector and/or such DNA molecules or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

In another aspect, the invention provides a recombinant expression vector comprising the nucleotide sequence of the human GABA receptor epsilon subunit together with additional sequences capable of directing the synthesis of the said human GABA receptor epsilon subunit in cultures of stably co-transfected eukaryotic cells.

The term "expression vectors" as used herein refers to DNA sequences that are required for the transcription of cloned copies of recombinant DNA sequences or genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, blue-green algae, yeast cells, insect cells, plant cells and animal cells. Specifically designed vectors allow the shuttling of DNA between bacteria-yeast, bacteria-plant or

bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

The term "cloning vector" as used herein refers to a DNA molecule, usually a small plasmid or bacteriophage DNA capable of self-replication in a host organism, and used to introduce a fragment of foreign DNA into a host cell. The foreign DNA combined with the vector DNA constitutes a recombinant DNA molecule which is derived from recombinant technology. Cloning vectors may include plasmids, bacteriophages, viruses and cosmids.

The recombinant expression vector in accordance with the invention may be prepared by inserting the nucleotide sequence of the GABA epsilon subunit into a suitable precursor expression vector (hereinafter referred to as the "precursor vector") using conventional recombinant DNA methodology known from the art. The precursor vector may be obtained commercially, or constructed by standard techniques from known expression vectors. The precursor vector suitably contains a selection marker, typically an antibiotic resistance gene, such as the neomycin or ampicillin resistance gene. The precursor vector preferably contains a neomycin resistance gene, adjacent the SV40 early splicing and polyadenylation region; an ampicillin resistance gene; and an origin of replication, e.g. pBR322 ori. The vector also preferably contains an inducible promoter, such as MMTV-LTR (inducible with dexamethasone) or metallothionin (inducible with zinc), so that transcription can be controlled in the cell line of this invention. This reduces or avoids any

problem of toxicity in the cells because of the chloride channel intrinsic to the $GABA_A$ receptor.

One suitable precursor vector is pMAMneo, available from Clontech Laboratories Inc. (Lee *et al., Nature*, 1981, 294, 228; and Sardet *et al., Cell,* 1989, 56, 271). Alternatively the precursor vector pMSGneo can be constructed from the vectors pMSG and pSV2neo.

The recombinant expression vector of the present invention is then produced by cloning the GABA receptor epsilon subunit cDNA into the above precursor vector. The receptor subunit cDNA is subcloned from the vector in which it is harboured, and ligated into a restriction enzyme site, e.g. the Hind III site, in the polylinker of the precursor vector, for example pMAMneo or pMSGneo, by standard cloning methodology known from the art, and in particular by techniques analogous to those described herein. Before this subcloning, it is often advantageous, in order to improve expression, to modify the end of the epsilon subunit cDNA with additional 5' untranslated sequences, for example by modifying the 5' end of the epsilon subunit DNA by addition of 5' untranslated region sequences from the α_1 subunit DNA.

According to a further aspect of the present invention, there is provided a stably co-transfected eukaryotic cell line capable of expressing a GABA receptor, which receptor comprises the epsilon receptor subunit, at least one alpha receptor subunit and at least one beta receptor subunit.

This is achieved by co-transfecting cells with three expression vectors, each harbouring cDNAs encoding for an α, β or ε GABA receptor subunit. In a further aspect, therefore, the present invention provides a process for the preparation of a eukaryotic cell line capable of expressing a GABA receptor, which comprises stably co-transfecting a eukaryotic host cell with at least three expression vectors, one such vector harbouring the cDNA sequence encoding the epsilon GABA receptor subunit, another such vector harbouring the cDNA sequence encoding an alpha GABA receptor subunit, and a third such vector harbouring the cDNA sequence

encoding the beta GABA receptor subunit. The stable cell-line which is established expresses an αβε GABA receptor.

Each receptor thereby expressed, comprising a unique combination of α, β and ε subunits, will be referred to hereinafter as a GABA receptor "subunit combination". Pharmacological and electrophysiological data confirm that the recombinant αβε receptor expressed by the cells of the present invention has the properties expected of a native GABA receptor.

Expression of the GABA receptor may be accomplished by a variety of different promoter-expression systems in a variety of different host cells. The eukaryotic host cells suitably include yeast, insect and mammalian cells. Preferably the eukaryotic cells which can provide the host for the expression of the receptor are mammalian cells. Suitable host cells include rodent fibroblast lines, for example mouse Ltk$^-$, Chinese hamster ovary (CHO) and baby hamster kidney (BHK); HeLa; and HEK293 cells. It is necessary to incorporate at least one α subunit, at least one β and the ε subunit into the cell line in order to produce the required receptor. Within this limitation, the choice of receptor subunit combination is made according to the type of activity or selectivity which is being screened for.

In order to employ this invention most effectively for screening purposes, it is preferable to build up a library of cell lines, each with a different combination of subunits. Typically a library of 5 or 6 cell line types is convenient for this purpose. Preferred subunit combinations include: $\alpha_1\beta_1\varepsilon$. Another preferred subunit combination is $\alpha_1\beta_2\varepsilon$.

Cells are then co-transfected with the desired combination of three expression vectors. There are several commonly used techniques for transfection of eukaryotic cells *in vitro*. Calcium phosphate precipitation of DNA is most commonly used (Bachetti *et al., Proc. Natl. Acad. Sci. USA*, 1977, 74, 1590-1594; Maitland *et al., Cell*, 1977, 14, 133-141), and represents a favoured technique in the context of the present invention.

A small percentage of the host cells takes up the recombinant DNA. In a small percentage of those, the DNA will integrate into the host cell chromosome. Because the neomycin resistance gene will have been incorporated into these host cells, they can be selected by isolating the individual clones which will grow in the presence of neomycin. Each such clone is then tested to identify those which will produce the receptor. This is achieved by inducing the production, for example with dexamethasone, and then detecting the presence of receptor by means of radioligand binding.

Alternatively, expression of the GABA receptor may be effected in *Xenopus* oocytes (see, for instance, Hadingham *et al. Mol. Pharmacol.*, 1993, 44, 1211-1218). Briefly, isolated oocyte nuclei are injected directly with injection buffer or sterile water containing at least one alpha subunit, at least one beta subunit, and the epsilon subunit engineered into a suitable expression vector. The oocytes are then incubated.

The expression of subunit combinations in the transfected oocytes may be demonstrated using conventional patch clamp assay. This assay measures the charge flow into and out of an electrode sealed on the surface of the cell. The flow of chloride ions entering the cell *via* the GABA gate ion channel is measured as a function of the current that leaves the cell to maintain electrical equilibrium within the cell as the gate opens.

In a further aspect, the present invention provides protein preparations of GABA receptor subunit combinations, especially human GABA receptor subunit combinations, derived from cultures of stably transfected eukaryotic cells of the present invention.

The protein preparations of GABA receptor subunit combinations can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity

chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The GABA epsilon subunit polypeptide of the present invention is also useful for identifying other subunits of the GABA receptor. An example of a procedure for identifying these subunits comprises raising high titre polyclonal antisera against unique, bacterially expressed GABA epsilon polypeptides. These polyclonal antisera are then used to immunoprecipitate detergent-solubilized GABA receptors from a mammalian brain, for example, a rat brain.

The invention also provides preparations of membranes containing subunit combinations of the GABA receptor, especially human GABA receptor subunit combinations, derived from cultures of stably transfected eukaryotic cells of the present invention.

The cell line, and the membrane preparations therefrom, according to the present invention have utility in screening and design of drugs which act upon the GABA receptor, for example benzodiazepines, barbiturates, β-carbolines and neurosteroids.

Receptor localisation studies using *in situ* hybridization in monkey brains shows that the ε subunit has a very restricted localisation; residing mainly in the hypothalamus and the arcuate nucleus. Weak expression

was also seen in the paraventricular nucleus and in the hilus of the hippocampus. This discrete distribution implies a possible role in the modulation of appetite behaviours (for example, hunger, thirst, sex) and possible hormonal interactions (vasopressin, oxytocin, adrenocorticotrophin hormone and gonadotrophins) and in the modulation of cognition.

The present invention accordingly provides the use of stably cotransfected cell lines described above, and membrane preparations derived therefrom, in screening for and designing medicaments which act upon GABA receptors comprising the ε subunit. Of particular interest in this context are molecules capable of interacting selectively with GABA receptors made up of varying subunit combinations. As will be readily apparent, the cell line in accordance with the present invention, and the membrane preparations derived therefrom, provide ideal systems for the study of structure, pharmacology and function of the various GABA receptor subtypes. In particular, preferred screens are functional assays utilizing the pharmacological properties of the GABA receptor subunit combinations of the present invention.

Thus, according to a further aspect of the present invention, there is provided a method for determining whether a ligand, not known to be capable of binding to a human $GABA_A$ receptor comprising the epsilon subunit, can bind to a human $GABA_A$ receptor comprising the epsilon subunit, which comprises contacting a mammalian cell comprising DNA molecules encoding at least one alpha receptor subunit, at least one beta receptor subunit and the epsilon receptor subunit with the ligand under conditions permitting binding of ligands known to bind to the $GABA_A$ receptor, detecting the presence of any of the ligand bound to the $GABA_A$ receptor comprising the epsilon subunit, and thereby determining whether the ligand binds to the $GABA_A$ receptor comprising the epsilon subunit. The epsilon subunit-encoding DNA in the cell may have a coding sequence substantially the same as the coding sequence shown in Figure 1.

Preferably, the mammalian cell is non-neuronal in origin. An example of a non-neuronal mammalian cell is a fibroblast cell such as an Ltk^- cell. The preferred method for determining whether a ligand is capable of binding to a human $GABA_A$ receptor comprising the epsilon subunit comprises contacting a transfected non-neuronal mammalian cell (i.e. a cell that does not naturally express any type of $GABA_A$ receptor, and thus will only express such a receptor if it is transfected into the cell) expressing a $GABA_A$ receptor comprising the epsilon subunit on its surface, or contacting a membrane preparation from such a transfected cell, with the ligand under conditions which are known to prevail, and thus to be associated with, *in vivo* binding of the ligands to a $GABA_A$ receptor comprising the epsilon subunit, detecting the presence of any of the ligand being tested bound to the $GABA_A$ receptor comprising the epsilon subunit on the surface of the cell, and thereby determining whether the ligand binds to a human $GABA_A$ receptor comprising the epsilon subunit. This response system may be based on ion flux changes measured, for example, by scintillation counting (where the ion is radiolabelled) or by interaction of the ion with a fluorescent marker. Particularly suitable ions are chloride ions. Such a host system is conveniently isolated from pre-existing cell lines. Such a transfection system provides a complete response system for investigation or assay of the activity of human $GABA_A$ receptors comprising the epsilon subunit with ligands as described above. Transfection systems are useful as living cell cultures for competitive binding assays between known or candidate drugs and ligands which bind to the receptor and which are labeled by radioactive, spectroscopic or other reagents. Membrane preparations containing the receptor isolated from transfected cells are also useful for these competitive binding assays. A transfection system constitutes a "drug discovery system" useful for the identification of natural or synthetic compounds with potential for drug development that can be further modified or used directly as therapeutic compounds to activate, inhibit or

modulate the natural functions of human $GABA_A$ receptors comprising the epsilon subunit. The transfection system is also useful for determining the affinity and efficacy of known drugs at human $GABA_A$ receptor sites comprising the epsilon subunit.

This invention also provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a human $GABA_A$ receptor comprising the epsilon subunit on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding at least one alpha receptor subunit, at least one beta receptor subunit, and the epsilon receptor subunit on the surface of a cell with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, human $GABA_A$ receptors comprising the epsilon subunit. The epsilon subunit-encoding DNA in the cell may have a coding sequence substantially the same as the coding sequence shown in Figure 1. Preferably, the mammalian cell is non-neuronal in origin. An example of a non-neuronal mammalian cell is a fibroblast cell such as an Ltk^- cell. Drug candidates are identified by choosing chemical compounds which bind with high affinity to the expressed $GABA_A$ receptor protein in transfected cells, using radioligand binding methods well known in the art. Drug candidates are also screened for selectivity by identifying compounds which bind with high affinity to one particular $GABA_A$ receptor combination but do not bind with high affinity to any other $GABA_A$ receptor combination or to any other known receptor site. Because selective, high affinity compounds interact primarily with the target $GABA_A$ receptor site after administration to the patient, the chances of producing a drug with unwanted side effects are minimized by this approach.

Ligands or drug candidates identified above may be agonists or antagonists at human $GABA_A$ receptors comprising the epsilon subunit, or may be agents which allosterically modulate a human $GABA_A$ receptor

comprising the epsilon subunit. These ligands or drug candidates identified above may be employed as therapeutic agents, for example, for the modulation of appetite behaviours, hormonal interactions and cognition.

The ligands or drug candidates of the present invention thus identified as therapeutic agents may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the agonist or antagonist, and a pharmaceutically acceptable carrier or excipient.

Preferably the compositions containing the ligand or drug candidate identified according to the methods of the present invention are in unit dosage forms such as tablets, pills, capsules, wafers and the like. Additionally, the therapeutic agent may be presented as granules or powders for extemporaneous formulation as volume defined solutions or suspensions. Alternatively, the therapeutic agent may be presented in ready-prepared volume defined solutions or suspensions. Preferred forms are tablets and capsules.

For preparing solid compositions such as tablets, the principal active ingredient is mixed with a pharmaceutical carrier, e.g. conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical diluents, e.g. water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a non-toxic pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation composition is then subdivided into unit dosage forms of the type described above containing from 0.1 to about 500 mg of the active ingredient of the present invention. The tablets or pills of the

novel composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally include aqueous solutions, suitably flavoured syrups, aqueous or oil suspensions, and flavoured emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, peanut oil or soybean oil, as well as elixirs and similar pharmaceutical vehicles. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinyl-pyrrolidone or gelatin.

Compositions of the present invention may also be administered via the buccal cavity using conventional technology, for example, absorption wafers.

Compositions in the form of tablets, pills, capsules or wafers for oral administration are particularly preferred.

A minimum dosage level for the ligand or drug candidate identified according to the methods of the present invention is about 0.05mg per day, preferably about 0.5mg per day and especially about 2.5mg per day. A maximum dosage level for the ligand or drug candidate is about 3000mg per day, preferably about 1500mg per day and especially about 500mg per day. The compounds are administered on a regimen of 1 to 4 times daily, preferably once or twice daily, and especially once a day.

It will be appreciated that the amount of the therapeutic agent required for use therapy will vary not only with the particular compounds or compositions selected but also with the route of administration, the nature of the condition being treated, and the age and condition of the patient, and will ultimately be at the discretion of the patient's physician or pharmacist.

DESCRIPTION OF FIGURES

Figure 1: Nucleotide sequence for the epsilon subunit, together with its deduced amino acid sequence corresponding thereto.

Figure 2: Concentration-response curve for the inhibition of an EC_{50} concentration of GABA by increasing concentrations of zinc.

Figure 3: Effects of pentobarbital on oocytes expressing $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ $GABA_A$ receptors.
a) Potentiation of the response to a GABA EC_{20} by increasing concentrations of pentobarbital on oocytes expressing $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ $GABA_A$ receptors.
b) Direct activation of the $GABA_A$ receptor by increasing concentrations of pentobarbital on oocytes expressing $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ $GABA_A$ receptors.
Data represent mean ± sem of four individual concentration-response curves.

Figure 4: Effects of propofol and etomidate on e-containing receptors.
a) Potentiation of the response to a GABA EC_{20} by 10μM propofol on oocytes expressing $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ $GABA_A$ receptors.
b) Potentiation of the response to a GABA EC_{20} by etomidate

on oocytes expressing $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_1\varepsilon$, $\alpha_1\beta_2$ and $\alpha_1\beta_2\varepsilon$ $GABA_A$ receptors.

Data represent mean ± sem of at least four determinations.

The following non-limiting Examples illustrate the present invention.

EXAMPLE 1

ISOLATION AND SEQUENCING OF A cDNA ENCODING THE HUMAN $GABA_A$ RECEPTOR ε SUBUNIT

The Expressed Sequence Tag (EST) database was searched with $GABA_A$ receptor polypeptide amino acid sequences using the BLAST searching algorithm, and a number of EST sequences identified. Two of these R07883 and R49718, were investigated in more detail. R07883 contained sequences homologous to the TM1-TM3 spanning domain of other $GABA_A$ receptor subunits. R49718 contained sequences homologous to the TM4 domain of $GABA_A$ receptors, a putative stop codon and putative 3' untranslated region. Polymerase chain reaction (PCR) was performed to determine if the two ESTs encoded the same gene product. For PCR, a sense primer was generated from the R07883 sequence (5' ctgttggagtttggtgtgctcaac 3'), and an antisense primer from the R49718 sequence (5' accagctggtacctacaagttaag 3'). PCR was performed using standard conditions (Whiting *et al, Proc. Natl. Acad. Sci., USA*, 1990, 87, 9966) using human sub thalamic cDNA as a template. A single PCR product of approximately 400bp was obtained indicating that the two ESTs encoded sequences of the same gene product.

cDNA sequences 5' of the R07883 sequence were obtained by 5' anchored PCR using human brain Marathon cDNA cloning kit (Clontech) according to manufacturer's protocols. The nested antisense primers used were derived from the R07883 sequence (AS1, 5' catcgtggtcacggaagaagggac 3'; AS2, 5' gccaaaccgcctgctcacattgaa 3'). PCR products were subcloned into pMos vector (Amersham) using standard techniques, and sequenced using an Applied Biosystems 373 DNA sequencer and dye terminator chemistry. One of the PCR products was found to extend far enough to contain sequence encoding a putative initiating methionine and 5' untranslated region.

A full length cDNA was generated by PCR using primers derived from sequences in the 5' UT of the anchored PCR product, and the 3' UT sequences in R49718 (5' caggtggtgcggccgctctccgcggaaatgttgt 3' and 5'tccacagggcggccgctggtacctacaagttaag 3', both incorporating a NotI site for subcloning). The PCR product (1550bp) was subcloned into pCDNAI Amp (Invitrogen, San Diego, CA) and sequenced completely on both strands by primer walking. Sequence analysis was performed using Inherit (Applied Biosystems) and Genetics Computer Group (Univ. Wisconsin) computer programs.

The coding region contains 506 amino acids and has the structural motifs expected on a ligand gated ion channel subunit. Comparison with other ligand gated ion channel subunits indicates that it is most simliar to $GABA_A$ receptor subunits, the highest homology being with the γ_3 subunit (47% identity). However this sequence identity is sufficiently low as to indicate that the new subunit cannot be classified as a fourth γ subunit, but represents a novel class of subunit, classified as ε, within the GABA receptor gene family.

EXAMPLE 2

EXPRESSION IN *XENOPUS* OOCYTES

Adult female *Xenopus laevis* were anaesthetised by immersion in a 0.4% solution of 3-aminobenzoic acid ethylester for 30-45 mins (or until unresponsive). Ovary tissue was removed via a small abdominal incision and Stage V and Stage VI oocytes were isolated with fine forceps. After mild collagenase treatment to remove follicle cells (Type IA, 0.5 mg ml^{-1}, for 8 mins), the oocyte nuclei were directly injected with 10-20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7, filtered through nitro-cellulose) or sterile water containing different combinations of human GABA subunit cDNAs (20 ng ml^{-1}) engineered into the expression vector pCDM8 or pcDNAI/Amp. Following incubation for 24-72 hrs, oocytes were placed in a 50µl bath and perfused at 4-6 ml min^{-1} with modified Barth's medium (MBS) consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, at pH 7.5. Cells were impaled with two 1-3 MΩ electrodes containing 2 M KCl and voltage-clamped between -40 and -70 mV.

In all experiments, drugs were applied in the perfusate until the peak of the response was observed. Non-cumulative concentration-response curves to agonists were constructed allowing at least three minutes between each agonist application to prevent desensitization. Curves were fitted using a non-linear square-fitting program to the equation $f(x) = B_{MAX}/[1+(EC_{50}/x)^n]$ where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response and n is the Hill coefficient. The effects of $GABA_A$ receptor modulators were examined on control GABA EC_{20} responses with a preapplication time of 30 secs. It was possible to demonstrate the coassembly of α_1 and β_1 subunits with the ε subunit by determining the inhibition by zinc. While zinc had an IC_{50} of

0.29mM at $\alpha_1\beta_1$ receptors, its affinity for $\alpha_1\beta_1\varepsilon$ receptors was over 140 fold lower (IC_{50} of 41.9mM). These results are shown in Figure 2. This data clearly demonstrates that the ε subunit coassembles with α and β subunits to form a novel GABA receptor subtype with a unique pharmacology.

EXAMPLE 3

LOCALISATION OF THE ε SUBUNIT IN MONKEY BRAIN BY *IN SITU* HYBRIDISATION.

Antisense oligonucleotide probes to the human ε subunit sequence were generated on an Applied Biosystems Automated DNA synthesiser

Probe 1

5' TGT-GCC-CGC-CAA-CAT-CAG-GAA-GCT-TTT-GTG-TGC-CAG-ATT-GTC-ACC 3'

Probe 2

5'ACT-TCT-GCA-TGG-TCC-CCG-ATT-GTG-AGG-GCA-GTA-CCT-GGC-AGT-AGG 3'

Each oligonucleotide was 3'-end labelled with [^{35}S] deoxyadenosine 5'-(thiotriphosphate) in a 30:1 molar ratio of ^{35}S-isotope:oligonucleotide using terminal deoxynucleotidyl transferase for 15 min at 37°C in the reaction buffer supplied. Radiolabelled oligonucleotide was separated from unincorporated nucleotides using Sephadex G50 spin columns. The specific activities of the labelled probes in several labelling reactions varied from 1.2-2.3 x 10^9 cpm/mg. Monkey brains were removed and fresh frozen in 1 cm blocks. 12 μm sections were taken and fixed for *in situ* hybridisation. Hybridisation of the sections was carried out according to the method of Sirinathsingji and Dunnett (Imaging gene expression in neural graft; *Molecular Imaging in Neuroscience: A Practical Approach*, N.A. Sharif (ed), Oxford University Press, Oxford, pp43-70, 1993). Briefly,

sections were removed from alcohol, air dried and 5 x10^5 cpm of each ^{35}S-labelled probe in 100µl of hybridisation buffer was applied to each slide. Labelled "antisense" probe was also used in the presence of an excess (100x) concentration of unlabelled antisense probe to define non-specific hybridisation. Parafilm coverslips were placed over the sections which were incubated overnight (about 16 hr) at 37°C. Following hybridisation the sections were washed for 1 hr at 57°C in 1xSSC then rinsed briefly in 0.1 x SSC, dehydrated in a series of alcohols, air dried and exposed to Amersham Hyperfilm βmax X-ray film and the relative distribution of the mRNA assessed for a variety of brain regions.

The localisation appears to be very restricted; residing mainly in the hypothalamus and the arcuate nucleus. Weak expression could also be seen in the paraventricular nucleus and in the hilus of the hippocampus. There was no detectable message in the caudate, putamen, globus pallidus, dorsal thalamus, amygdala, brain stem or cerebellum. The discrete distribution (the most restricted of any known $GABA_A$ receptor subunit) implies a possible function in "appetite behaviours" (hunger, thirst, sex) and possible hormonal interactions (vasopressin, oxytocin, adrenocorticotrophin hormone and gonadotrophins).

EXAMPLE 4

PHARMACOLOGY OF αβε SUBUNIT COMBINATIONS

The effect of a number of benzodiazepines from a range of different chemical classes were examined on oocytes expressing $\alpha_1\beta_1\varepsilon$ receptors (Table 1). Several studies have shown that variation of the β subunit does not affect benzodiazepine modulation (Puia *et al. Natl. Acad. Sci. USA*, 1992, 89, 3620-3624; Hadingham *et al., Mol. Pharmacol.*, 1993, 44, 1211-1218) or binding (Pritchett *et al., Science*, 1989, 245, 1389-1392) and

therefore binding affinity and efficacy on $\alpha_1\beta_3\gamma_{2S}$ receptors are included as a comparison.

TABLE 1

Compound	$\alpha_1\beta_1\varepsilon$ efficacy (% modulation of GABA EC_{20})	$\alpha_1\beta_3\gamma_{2S}$ efficacy (% modulation of GABA EC_{20})	$\alpha_1\beta_3\gamma_{2S}$ Ki (nM)
flunitrazepam	0 ± 0	121 ± 9.3	2.2
bretazenil	-1 ± 1.5	29.3 ± 3.6	0.4
zolpidem	-5.8 ± 4.0	134 ± 17	26.7
CL-218,872	-0.8 ± 5.4	71 ± 17	57
abecarnil	-5.0 ± 5.9	74 ± 20	12.4
FG8205	-1.3 ± 2.4	86 ± 9	0.4
SX-3228	-3 ± 1.1	77.0 ± 5.9	4.5

$\alpha_1\beta_1\varepsilon$: all benzodiazepines tested at 1μM except CL-218872 which was tested at 10μM.

$\alpha_1\beta_3\gamma_{2S}$: all benzodiazepines tested at approximately 100 x their Ki.

Data shown are the mean ± sem of four determinations.

The results demonstrate that $\alpha_1\beta_1\varepsilon$ receptors are not modulated by benzodiazepines.

A number of intravenous anaesthetic agents have been shown to both modulate and directly activate $GABA_A$ receptors. Concentration-response curves for the potentiation of control GABA EC_{20} currents to pentobarbital were produced (Figure 3). A maximum potentiation of control GABA EC_{20} currents of 384 ± 41% on $\alpha_1\beta_1$, 275 ± 17% on $\alpha_1\beta_1\gamma_{2S}$ and 233 ± 31% on $\alpha_1\beta_1\varepsilon$ was observed. The EC_{50} and slope, of approximately 30μM and 1.5 respectively, were similar for all three receptor combinations.

Pentobarbital will directly activate the $GABA_A$ receptor at concentrations higher than those required to cause potentiation. This GABA mimetic effect by pentobarbital was examined on $\alpha_1\beta_1\varepsilon$ receptors and compared to $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors. The maximum activation by pentobarbital as a percentage of the maximum GABA response was 60% on $\alpha_1\beta_1$ compared to 33% on $\alpha_1\beta_1\beta_{2S}$ and 27% on $\alpha_1\beta_1\varepsilon$. The EC_{50} was lowest on $\alpha_1\beta_1\varepsilon$ (187 (122; 287)μM) followed by $\alpha_1\beta_1$ (335 (307; 365)μM) and then $\alpha_1\beta_1\gamma_{2S}$ (540 (476; 611)μM). The Hill coefficient was significantly lower for $\alpha_1\beta_1\varepsilon$ receptors (1.8 ± 0.1) than on $\alpha_1\beta_1$ receptors (4.8± 0.9) or $\alpha_1\beta_1\gamma_{2S}$ receptors (3.3 ± 0.3). A consequence of the lower Hill coefficient and higher affinity for $\alpha_1\beta_1\varepsilon$ receptors is direct activation by pentobarbital at lower concentrations on $\alpha_1\beta_1\varepsilon$ receptors than on either $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors.

Two other anaesthetics, propofol and etomidate, were examined on $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ receptors (Figure 4). Potentiation of GABA EC_{20} currents by 10μM propofol was 277 ± 62% for $\alpha_1\beta_1\varepsilon$ receptors and 214 ± 9% for $\alpha_1\beta_1\gamma_{2S}$ receptors both of which were lower than that obtained on $\alpha_1\beta_1$ receptors (472 ± 108%). Etomidate (30μM) also potentiated $\alpha_1\beta_1$ receptors to a greater extent than $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\varepsilon$ receptors (419 ± 102% verses 218 ± 30% and 230 ±17% respectively). Etomidate potentiation has been shown to depend on the β subunit isoform (Hill-Venning *et al.*, *Br. J. Pharmacol.*, 1997, 120, 749-756). When the β_2 isoform is coexpressed with $\alpha_1\varepsilon$ a 10 fold increase in etomidate potency is observed compared to $\alpha_1\beta_1\varepsilon$. These results demonstrate that substitution of the γ subunit with the ε subunit does not alter the β subunit selectivity with etomidate. Small direct effects were observed with both propofol and etomidate on $\alpha_1\beta_1\varepsilon$ receptors which were absent on $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors. This correlates with the higher potency for direct activation observed with pentobarbital.

CLAIMS:

1. A stably co-transfected eukaryotic cell line capable of expressing a GABA receptor, which receptor comprises the eplison receptor subunit, at least one alpha receptor subunit and at least one beta receptor subunit.

2. A cell line according to claim 1 which is a rodent fibroblast cell line.

3. A process for the preparation of an eukaryotic cell line capable of expressing a GABA receptor, which comprises stably co-transfecting a eukaryotic host cell with at least three expression vectors, one such vector harbouring the cDNA sequence encoding the epsilon GABA receptor subunit, another such vector harbouring the cDNA sequence encoding an alpha GABA receptor subunit, and a third such vector harbouring the cDNA sequence encoding a beta GABA receptor subunit.

4. A process according to claim 3 wherein the cell line is a rodent fibroblast cell line.

5. A DNA molecule encoding the epsilon subunit of the human GABA receptor comprising all or a portion of the sequence depicted in Figure 1, or a modified human sequence.

6. A recombinant expression vector comprising the nucleotide sequence of the human GABA receptor epsilon subunit together with additional sequences capable of directing the synthesis of the said human GABA receptor epsilon subunit in cultures of stably co-transfected eukaryotic cells.

7. A protein preparation of GABA receptor subunit combinations derived from a cell line according to claim 1 or 2.

8. A membrane preparation containing subunit combinations of the GABA receptor derived from a cell line according to claim 1 or 2.

9. A preparation according to claim 7 or 8 wherein the subunit combination derived is the $\alpha_1\beta_1\varepsilon$ subunit combination of the GABA receptor.

10. A preparation according to claim 7 or 8 wherein the subunit combination derived is the $\alpha_1\beta_2\varepsilon$ subunit combination of the GABA receptor.

11. The use of a cell according to claim 1 or 2 or a membrane preparation derived therefrom in screening for and designing medicaments which act upon a GABA receptor comprising the ε subunit.

12. A method for determining whether a ligand, not known to be capable of binding to a human $GABA_A$ receptor comprising the epsilon subunit, can bind to a human $GABA_A$ receptor comprising the epsilon subunit, which comprises contacting a mammalian cell comprising DNA molecules encoding at least one alpha receptor subunit, at least one beta receptor subunit and the epsilon receptor subunit with the ligand under conditions permitting binding of ligands known to bind to the $GABA_A$ receptor, detecting the presence of any of the ligand bound to the $GABA_A$ receptor comprising the epsilon subunit and thereby determining whether the ligand binds to the $GABA_A$ receptor comprising the epsilon subunit.

13. A method of screening drugs to identify drugs which specifically interact with, and bind to, a human $GABA_A$ receptor comprising the

epsilon subunit on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding at least one alpha receptor subunit, at least one beta receptor subunit, and the epsilon receptor subunit on the surface of a cell with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, human $GABA_A$ receptors comprising the epsilon subunit.

14. A polynucleotide which hybridizes under stringent conditions to the DNA molecule depicted in Figure 1.

15. A $GABA_A$ receptor epsilon subunit polypeptide which has the deduced amino acid sequence of Figure 1, or a fragment, analog or derivative thereof.

FIGURE 1

Human ε Subunit

```
   1 GCGGCCGCTCCGCGGAAATGTTGTCCAAAGTTCTTCCAGTCCTCCTAGGCATCTTATTGA 60
                     M  L  S  K  V  L  P  V  L  L  G  I  L  L  I

  61 TCCTCCAGTCGAGGGTCGAGGGACCTCAGACTGAATCAAAGAATGAAGCCTCTTCCCGTG 120
      L  Q  S  R  V  E  G  P  Q  T  E  S  K  N  E  A  S  S  R  D

 121 ATGTTGTCTATGGCCCCCAGCCCCAGCCTCTGGAAAATCAGCTCCTCTCTGAGGAAACAA 180
      V  V  Y  G  P  Q  P  Q  P  L  E  N  Q  L  L  S  E  E  T  K

 181 AGTCAACTGAGACTGAGACTGGGAGCAGAGTTGGCAAACTGCCAGAAGCCTCTCGCATCC 240
      S  T  E  T  E  T  G  S  R  V  G  K  L  P  E  A  S  R  I  L

 241 TGAACACTATCCTGAGTAATTATGACCACAAACTGCGCCCTGGCATTGGAGAGAAGCCCA 300
      N  T  I  L  S  N  Y  D  H  K  L  R  P  G  I  G  E  K  P  T

 301 CTGTGGTCACTGTTGAGATCTCCGTCAACAGCCTTGGTCCTCTCTCTATCCTAGACATGG 360
      V  V  T  V  E  I  S  V  N  S  L  G  P  L  S  I  L  D  M  E

 361 AATACACCATTGACATCATCTTCTCCCAGACCTGGTACGACGAACGCCTCTGTTACAACG 420
      Y  T  I  D  I  I  F  S  Q  T  W  Y  D  E  R  L  C  Y  N  D

 421 ACACCTTTGAGTCTCTTGTTCTGAATGGCAATGTGGTGAGCCAGCTATGGATCCCGGACA 480
      T  F  E  S  L  V  L  N  G  N  V  V  S  Q  L  W  I  P  D  T

 481 CCTTTTTTAGGAATTCTAAGAGGACCCACGAGCATGAGATCACCATGCCCAACCAGATGG 540
      F  F  R  N  S  K  R  T  H  E  H  E  I  T  M  P  N  Q  M  V

 541 TCCGCATCTACAAGGATGGCAAGGTGTTGTACACAATTAGGATGACCATTGATGCCGGAT 600
      P  I  Y  K  D  G  K  V  L  Y  T  I  R  M  T  I  D  A  G  C

 601 GCTCACTCCACATGCTCAGATTTCCAATGGATTCTCACTCTTGCCCTCTATCTTTCTCTA 660
      S  L  H  M  L  R  F  P  M  D  S  H  S  C  P  L  S  F  S  S

 661 GCTTTTCCTATCCTGAGAATGAGATGATCTACAAGTGGGAAAATTTCAAGCTTGAAATCA 720
      F  S  Y  P  E  N  E  M  I  Y  K  W  E  N  F  K  L  E  I  N

 721 ATGAGAAGAACTCCTGGAAGCTCTTCCAGTTTGATTTTACAGGAGTGAGCAACAAAACTG 780
      E  K  N  S  W  K  L  F  Q  F  D  F  T  G  V  S  N  K  T  E

 781 AAATAATCACAACCCCAGTTGGTGACTTCATGGTCATGACGATTTTCTTCAATGTGAGCA 840
      I  I  T  T  P  V  G  D  F  M  V  M  T  I  F  F  N  V  S  R

 841 GGCGGTTTGGCTATGTTGCCTTTCAAAACTATGTCCCTTCTTCCGTGACCACGATGCTCT 900
      R  F  G  Y  V  A  F  Q  N  Y  V  P  S  S  V  T  T  M  L  S

 901 CCTGGGTTTCCTTTTGGATCAAGACAGAGTCTGCTCCAGCCCGGACCTCTCTAGGGATCA 960
      W  V  S  F  W  I  K  T  E  S  A  P  A  R  T  S  L  G  I  T

 961 CCTCTGTTCTGACCATGACCACGTTGGGCACCTTTTCTCGTAAGAATTTCCCGCGTGTCT 1020
      S  V  L  T  M  T  T  L  G  T  F  S  R  K  N  F  P  R  V  S

1021 CCTATATCACAGCCTTGGATTTCTATATCGCCATCTGCTTCGTCTTCTGCTTCTGCGCTC 1080
      Y  I  T  A  L  D  F  Y  I  A  I  C  F  V  F  C  F  C  A  L

1081 TGTTGGAGTTTGCTGTGCTCAACTTCCTGATCTACAACCAGACAAAAGCCCATGCTTCTC 1140
      L  E  F  A  V  L  N  F  L  I  Y  N  Q  T  K  A  H  A  S  P

1141 CTAAACTCCGCCATCCTCGTATCAATAGCCGTGCCCATGCCCGTACCCGTGCACGTTCCC 1200
      K  L  R  H  P  R  I  N  S  R  A  H  A  R  T  R  A  R  S  R

1201 GAGCCTGTGCCCGCCAACATCAGGAAGCTTTTGTGTGCCAGATTGTCACCACTGAGGGAA 1260
      A  C  A  R  Q  H  Q  E  A  F  V  C  Q  I  V  T  T  E  G  S
```

```
1261 GTGATGGAGAGGAGCGCCCGTCTTGCTCTGCCCAGCAGCCCCCTAGCCCAGGTAGCCCTG 1320
       D  G  E  E  R  P  S  C  S  A  Q  Q  P  P  S  P  G  S  P  E

1321 AGGGTCCCCGCAGCCTCTGCTCCAAGCTGGCCTGCTGTGAGTGGTGCAAGCGTTTTAAGA 1380
       G  P  R  S  L  C  S  K  L  A  C  C  E  W  C  K  R  F  K  K

1381 AGTACTTCTGCATGGTCCCCGATTGTGAGGGCAGTACCTGGCAGCAGGGCCGCCTCTGCA 1440
       Y  F  C  M  V  P  D  C  E  G  S  T  W  Q  Q  G  R  L  C  I

1441 TCCATGTCTACCGCCTGGATAACTACTCGAGAGTTGTTTTCCCAGTGACTTTCTTCTTCT 1500
       H  V  Y  R  L  D  N  Y  S  R  V  V  F  P  V  T  F  F  F  F

1501 TCAATGTGCTCTACTGGCTTGTTTGCCTTAACTTGTAGGTACCAGCGGCCGC 1552
       N  V  L  Y  W  L  V  C  L  N  L
```

FIGURE 2

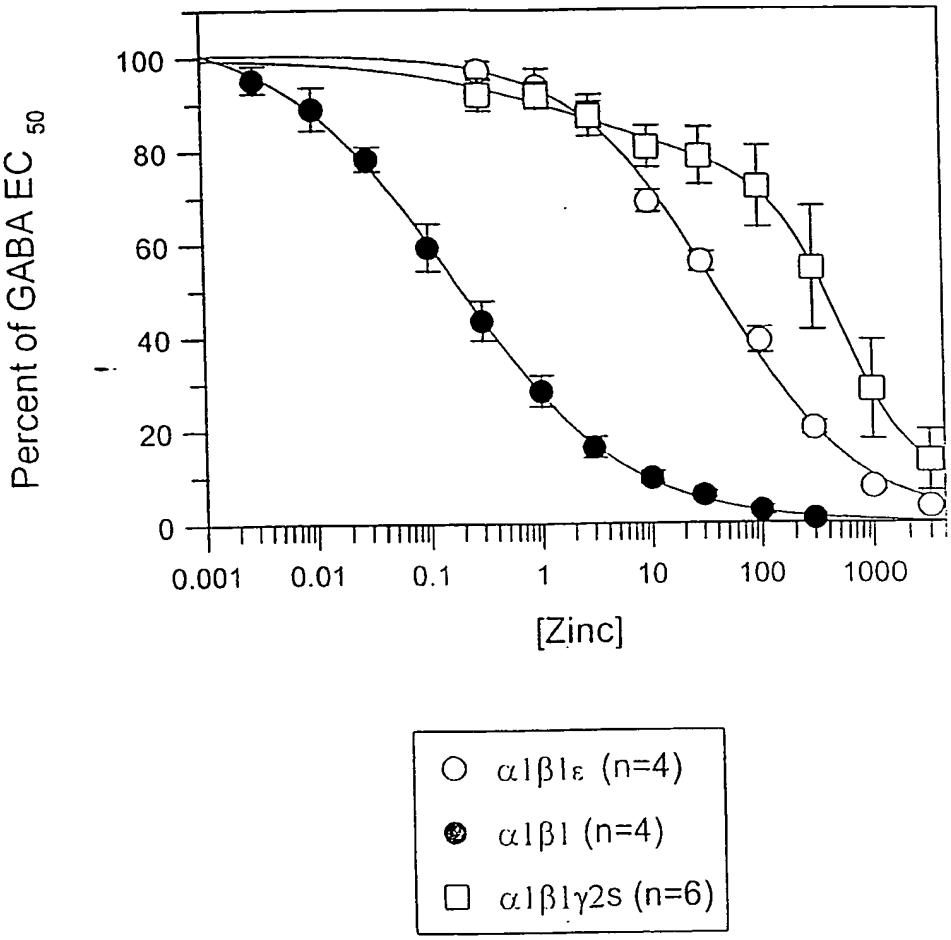
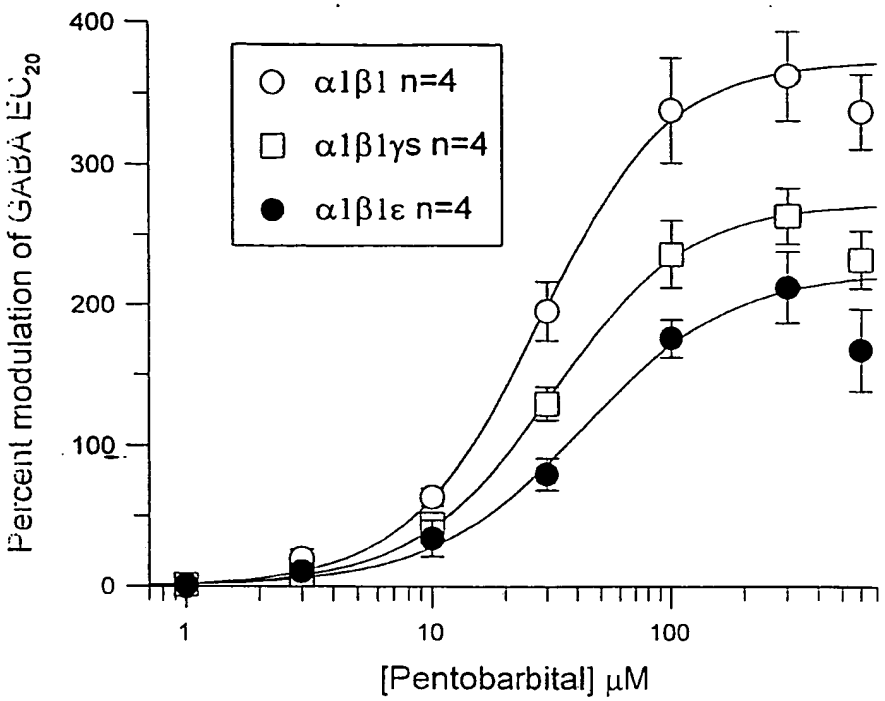

Percent of GABA EC 50
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
[Zinc]
α1β1ε (n=4)
α1β1 (n=4)
α1β1γ2s (n=6)

FIGURE 3


Percent modulation of GABA EC20
400
300
200
100
0
α1β1 n=4
α1β1γs n=4
α1β1ε n=4
1
10
100
[Pentobarbital] μM

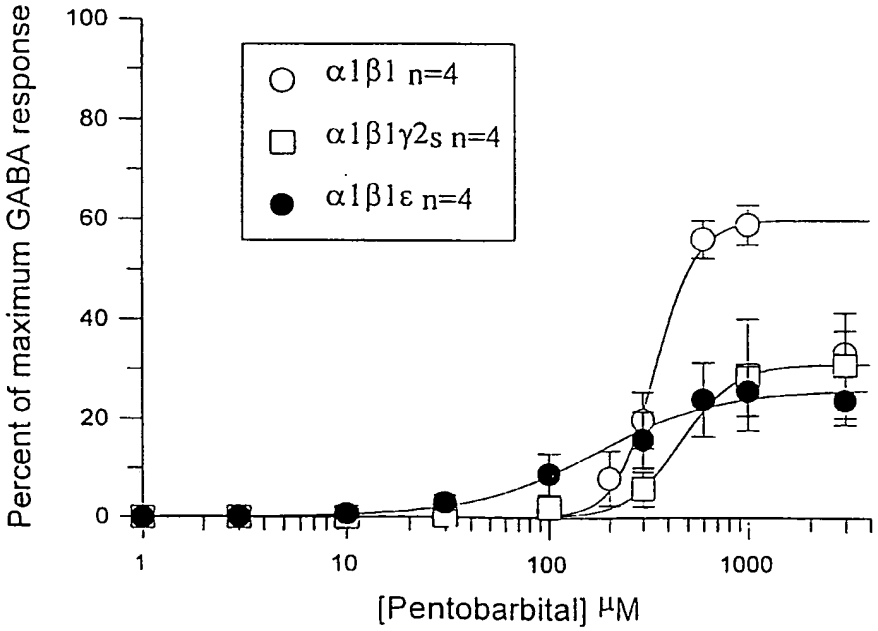
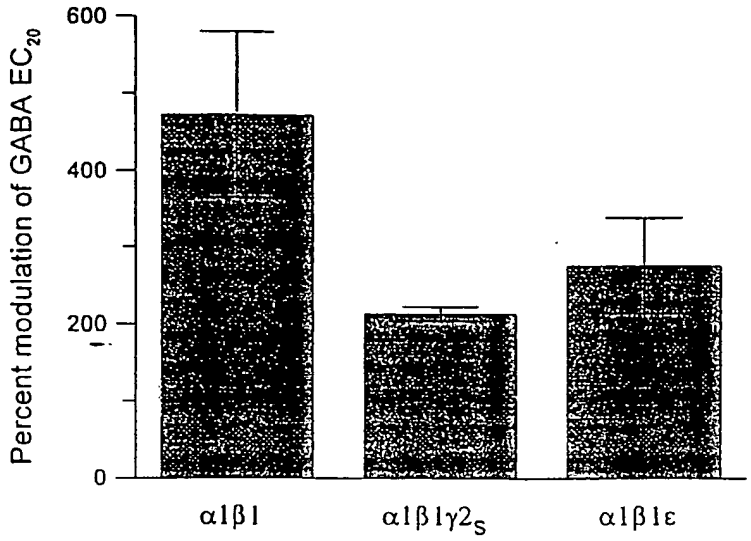

Percent of maximum GABA response
100
80
60
40
20
0
α1β1 n=4
α1β1γ2s n=4
α1β1ε n=4
1
10
100
1000
[Pentobarbital] μM

FIGURE 4

(a) Potentiation of GABA EC_{20} by 10μM propofol



(b) Potentiation of GABA EC_{20} by etomidate